Frequent HIN-1 promoter methylation and lack of expression in multiple human tumor types

Running title: HIN-1 silencing in multiple tumor types

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Abstract

HIN-1 is a candidate tumor suppressor identified as a gene silenced by methylation in the majority of breast carcinomas. HIN-1 is highly expressed in the mammary gland, trachea, lung, prostate, pancreas, and salivary gland, and in the lung its expression is mostly restricted to bronchial epithelial cells. In this report we demonstrate that correlating with the secretory nature of HIN-1, high levels of HIN-1 protein are detected in bronchial lavage, saliva, plasma, and serum. To determine if, similar to breast carcinomas, HIN-1 is also silenced in tumors originating from other organs with high HIN-1 expression, we analyzed its expression and promoter methylation status in lung, prostate, and pancreatic carcinomas. Nearly all prostate and a significant fraction of lung and pancreatic carcinomas demonstrated HIN-1 hypermethylation and the majority of lung and prostate tumors lacked HIN-1 expression. In lung carcinomas the degree of HIN-1 methylation differed among tumor subtypes ($p=0.02$) with the highest level of HIN-1 methylation observed in squamous cell carcinomas and the lowest in small cell lung cancer. In lung adenocarcinomas the expression of HIN-1 correlated with cellular differentiation status. Thus, silencing of HIN-1 expression and methylation of its promoter occurs in multiple human cancer types, suggesting that elimination of HIN-1 function may contribute to several forms of epithelial tumorigenesis.

Introduction

HIN-1 (High in Normal-1) was identified by SAGE (Serial Analysis of Gene Expression) analysis as a gene highly expressed in normal luminal mammary epithelial cells and downregulated in *in situ*, invasive, and metastatic breast carcinomas (1). The silencing of HIN-1 expression in the majority of breast tumors was found to be due to methylation of the proximal promoter and first exon of the HIN-1 gene (1). The high frequency of loss of HIN-1 expression in human breast carcinomas suggested a tumor suppressor function and correlating with this, reintroduction of HIN-1 into breast cancer cells inhibited cell growth (1). During mouse embryonic development the expression of HIN-1 was associated with the terminal differentiation of tracheo-bronchial epithelial cells (2). In addition, HIN-1 was up-regulated by retinoic acid induced differentiation of human bronchial epithelial cells suggesting a role for HIN-1 in mucinous epithelial cell differentiation (2). Correlating with this, a homologue of HIN-1, UGRP-1 (Uteroglobin related protein-1), was identified in the mouse as a target of the Nkx2.1 homeogene that is required for lung development and differentiation (3, 4). UGRP-1 was found to have a limited homology to uteroglobin, thus, both HIN-1 and UGRP-1 are considered to be distant members of the secretoglobin family and designated as secretoglobin 3A1 (SCGB3A1) and secretoglobin 3A2 (SCGB3A2), respectively.

To further dissect the role of HIN-1 in epithelial cell function and tumorigenesis, we analyzed its expression in various normal human organs and body fluids. In addition, we determined HIN-1 expression and promoter methylation status in lung, prostate, and pancreatic tumors.

Results and discussion

HIN-1 in normal lung tissue and body fluids

We have previously determined that human HIN-1 is highly expressed in the adult mammary gland, lung, trachea, pancreas, prostate and salivary gland (1). In mouse the highest HIN-1 expression is detected in the lung with much lower levels observed in other organs including the mammary gland (2, 3, 5). In the mouse lung the HIN-1 message is localized to the epithelial cells of the trachea, bronchi and bronchioli and during embryonic development HIN-1 mRNA levels correlate with the terminal differentiation of these cells (2). To determine the expression of HIN-1 in human lung tissue at the cellular level we performed mRNA in situ hybridization analysis. As depicted in Fig. 1A, similar to mouse, the HIN-1 message is specifically localized to bronchial epithelial cells, while lower levels are detected in some pneumocytes in the lung parenchyma. No HIN-1 expression was detected in type I epithelial cells of the distal alveolar sacs consistent with proximal muco-ciliary and distal alveolar epithelial cells being derived from a different lineage during development (6), although some reactive type II epithelial cells showed sparse HIN-1 expression (Fig. 1A). In addition, not all proximal bronchial epithelial cells expressed HIN-1, but it is currently unknown if HIN-1 expressing cells represent a specific cellular subtype.

Since HIN-1 is a secreted protein the high expression in epithelial cells lining the bronchi suggested that HIN-1 may be secreted into the bronchial lumen and therefore, could be detected in bronchial wash fluid. In order to test this hypothesis we performed immunoprecipitation followed by immunoblot analysis of bronchial wash fluid obtained from several individuals. HIN-1 protein was highly abundant in all three samples analyzed (Fig. 1B). To determine if HIN-1 is detected in other body fluids we also analyzed saliva, plasma, and serum from several healthy individuals. The salivary gland expresses high levels of HIN-1 mRNA, therefore, not surprisingly HIN-1 protein was highly abundant in all saliva samples (Fig. 1B). The high HIN-1 protein level detected in the blood was

somewhat unexpected and could be due to its non-polarized secretion by the epithelial cells that express it or its re-absorption through the gastrointestinal system. In all of these body fluids, under reducing conditions the endogenous HIN-1 protein migrated at its predicted molecular weight (~10 kDa), while in non-reducing conditions we detected a ~20kDa band presumably corresponding to a disulfide linked dimer (data not shown). Since proteases are abundant in both saliva and clotting blood, endogenous HIN-1 protein dimers must be fairly stable and resistant to proteases.

HIN-1 expression in lung and prostate tumors

To determine if, as observed in breast cancer, HIN-1 expression is lost or downregulated in lung carcinomas we performed Northern blot analysis of 27 lung adenocarcinomas and 12 squamous lung tumors. Most of the tumors showed no or very low levels of HIN-1 expression compared to normal lung tissue (data not shown). Since these tumors were not microdissected even the observed low level of HIN-1 expression could be due to contaminating normal bronchial epithelial cells. To evaluate the expression of HIN-1 in more detail we used mRNA in situ hybridization to determine the expression of HIN-1 at the cellular level. All 8 squamous tumors and 8 of 10 adenocarcinomas analyzed completely lacked HIN-1 expression, while the well but not the moderately differentiated parts of the remaining two adenocarcinomas showed high HIN-1 levels (Fig. 1C and data not shown). These results correlate well with our previous results demonstrating a link between HIN-1 expression and mucinous differentiation of bronchial epithelial cells (2).

The decreased HIN-1 expression in lung tumors could suggest that these tumors originate from a cell type that does not normally express HIN-1 or that its expression is silenced during tumorigenesis. Adenocarcinomas and squamous carcinomas are frequently localized to the distal and proximal lung, respectively, and they have distinct gene expression profiles (7-9) and genetic changes (10), and they are presumed to arise from different cell types (9, 11, 12).

To analyze HIN-1 expression in different parts of the normal prostate and in prostate tumors, we performed RT-PCR analysis of the left and right peripheral and central zones of normal prostate, a BPH (Benign Prostatic Hyperplasia) and seven prostate carcinomas. A high level of HIN-1 expression was detected in all zones of normal prostate and in BPH, while most prostate carcinomas lacked HIN-1 expression (Fig. 1D).

Methylation of HIN-1 in normal and cancerous lung, prostate, and pancreatic tissue and cell lines

In breast carcinomas we found a strong correlation between lack of HIN-1 expression and hypermethylation of its promoter region suggesting that methylation is responsible for silencing HIN-1 expression in tumors (1). To determine if HIN-1 promoter methylation also occurs in other cancer types that lack HIN-1 expression, we first analyzed the sequence of the HIN-1 proximal promoter and first exon in cancer cell lines, and normal and cancerous tissues from various organs. This analysis demonstrated that the HIN-1 promoter region is heavily methylated in two prostate cancer cell lines, while low and moderate level of methylation were seen in primary lung carcinomas and a pancreatic cancer cell line, respectively (Fig. 2A). None of the prostate and pancreatic cancer cell lines demonstrated significant levels of HIN-1 mRNA suggesting that loss of expression is associated with promoter methylation.

Some breast cancer cell lines demonstrated dense, while others lower levels of methylation. Since none of these cell lines expressed significant HIN-1 mRNA, methylation of other areas or methylation independent mechanisms may be responsible for the silencing of HIN-1 in some cells. Interestingly in normal breast tissue the most proximal promoter region (up to ~-400 bp) was completely unmethylated, while we detected some methylation in the distal (~-500 bp) promoter area in tissue from an 18 but not a 34 year-old patient suggesting interpersonal variability or possible correlation with age or reproductive history.

Based on the methylation and gene expression information obtained from the HIN-1 promoter sequence and RT-PCR analyses, respectively, the frequency of methylated CGs appeared to be higher at more distal (-120 to -300 bp) parts of the promoter in lung, prostate and pancreas compared to breast carcinomas and this correlated most consistently with lack of HIN-1 expression (Fig. 2A). Therefore, we designed new primers and developed a more distal HIN-1 promoter specific MSP (methylation specific PCR) assay (13) instead of using the one we have previously used for breast tumors (1). The newly designed primers gave nearly identical results to that of the previously published ones on a set of breast carcinomas (data not shown). Using this new MSP assay we analyzed the methylation status of HIN-1 in primary lung, prostate and pancreatic carcinomas, corresponding normal tissue, and cancer cell lines. Similar to breast carcinomas a significant fraction of lung, prostate, and pancreatic carcinomas showed complete or partial methylation (Table 1, Fig. 2). The highest frequency of HIN-1 methylation was observed in prostate cancer where all xenografts (9/9) and almost all (20/21) primary tumors were methylated. Similarly all pancreatic cancer cell lines (10/10) and a high fraction of primary tumors (9/17) demonstrated HIN-1 methylation (Table 1). Most importantly the HIN-1 promoter was highly methylated in all lung and prostate tumors that lacked HIN-1 expression based on in situ hybridization (Fig. 1C and data not shown) and RT-PCR (Fig. 1D), respectively. Thus, similar to breast carcinomas, HIN-1 promoter methylation appears to correlate with its lack of expression in lung and prostate carcinomas.

In contrast to results obtained in breast, a high fraction of histologically non-malignant prostate and pancreatic tissue samples collected from areas adjacent to tumors was also partially methylated (1). However, two BPH (Benign Prostatic Hyperplasia) samples obtained from bladder cancer patients and four normal lung tissue samples resected from hamartoma patients were all completely unmethylated (Fig. 2B and data not shown). Similar results were obtained by other groups using multiple normal human tissue types including lung, brain, blood, and bone ((14) and S. Sukumar personal communication). Therefore, the high frequency of HIN-1 methylation in the histologically non-

malignant tissue samples resected from cancer patients may indicate that there were contaminating tumor cells not noticed by microscopic examination or that even the normal appearing tissue of cancer patients already has acquired pre-malignant changes including HIN-1 promoter methylation. If this latter hypothesis is true then HIN-1 promoter methylation analysis from biopsy specimens may help in the identification of patients at high risk for malignancy.

In the case of lung the overall frequency of HIN-1 methylation was somewhat lower and varied according to cancer type. The lowest HIN-1 methylation frequency was observed in small cell lung cancer and the highest in squamous tumors (Table 1). The differences in HIN-1 methylation frequencies among the three lung cancer subtypes were statistically significant ($p=0.02$) suggesting that different lung tumors may originate from different cell types and/or have distinct tumorigenesis pathways. The finding that the highest level of HIN-1 methylation is found in squamous lung carcinomas is particularly interesting in light of the fact that these tumors are frequently proximal, as is HIN-1 expression in normal lung, and that in normal bronchial epithelial cells the expression of HIN-1 is down-regulated in cells that have lost their mucinous differentiation phenotype and acquired squamous features (2). Correlating with our HIN-1 methylation results, recent studies have described distinct clustering of lung and other carcinomas according to histologic subtypes based on the methylation profile of a set of genes known to be methylated in lung cancer and microarray based DNA methylation analysis, respectively (15, 16). Similar to prostate and pancreatic tumors, we found that a high fraction of corresponding non-malignant lung tissue was also methylated, but in all these cases the matched tumor showed even higher levels of methylation (Fig. 2B). Again, this suggests that the adjacent non-malignant tissue may have had some contaminating cancer cells or that premalignant changes have occurred in these adjacent tissues.

The observation that in lung adenocarcinomas the expression of HIN-1 correlated with the differentiation status of the cells (Fig. 1D) raised the question of whether down-regulation and methylation of HIN-1 simply reflects lack of differentiation or it is specifically associated with

tumorigenesis. To distinguish between these possibilities we analyzed the methylation status of the HIN-1 promoter in normal bronchial epithelial cells grown in the absence of retinoic acid resulting in loss of mucinous differentiation, acquisition of squamous characteristics, and lack of HIN-1 expression. No methylation was detected in these cells by MSP and sequencing analysis suggesting that lack of the normal mucinous differentiation program in these cells by itself is not sufficient to lead to HIN-1 methylation (Fig. 2A and data not shown).

In summary, these results demonstrate that silencing of HIN-1 expression due to methylation occurs in multiple human cancer types originating from organs that normally have high HIN-1 expression levels. In lung carcinomas HIN-1 methylation appears to be specifically associated with tumorigenesis and distinct histologic subtypes, potentially suggesting different cell type of origin or pathways of tumorigenesis for squamous, adeno-, and small cell lung carcinomas.

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Materials and methods

Cell lines and tissue specimens

All human tissue specimens were collected at the Brigham and Women's Hospital and National Institute of Health following NIH guidelines and using protocols approved by the Institutional Review Boards. Human cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) or were generously provided by Dr. Steve Ethier (University of Michigan Medical Center). Cells were grown in media recommended by the provider.

mRNA in situ hybridization and northern blot analysis

To generate templates for in vitro transcription reactions the full-length human HIN-1 cDNA was PCR amplified and subcloned into pZERO 1.0 (Invitrogen, Carlsbad, CA) and was used for the generation of sense and anti-sense digitonin-labeled riboprobes followed by mRNA *in situ* hybridizations essentially as previously described (17). The hybridized sections were observed with a NIKON microscope, images were obtained using a Axio Camera, and photographs were organized using the AxioVision software. Hybridizations were considered successful if the sense probe gave no significant signal. Northern blot analysis was performed as previously described (1).

Immunoprecipitation and immuno blot analysis

For immunoprecipitation analysis human body fluids were supplemented with salt and reagents to 500mM NaCl, 10% glycerol, 20mM Tris pH 8, and 0.5% Triton X-100 final concentration and incubated with pre-immune serum or anti-HIN-1 antibody coupled to proteinA-sepharose beads for two hours at 4°C followed by repeated washes in the same buffer. Immunocomplexes were resolved on 12% SDS NuPAGE gels (Invitrogen, Carlsbad, CA) and immunoblotted with anti-HIN-1 antibody as previously described (1).

Methylation assays and statistical analysis

Genomic DNA preparations and bisulfite treatment were performed as described previously (1). PCR primers used were as follows: For methylation specific PCRs: unmethylated: forward primer 5'-ATTGTAAAGTGAAGGTGTGGGTT-3', reverse primer 5'-CCAACTTCCTACTACAACCAACA-3'; Methylated: forward primer 5'-GTTTAGTTTTGAGGGGGGCGC-3', reverse primer 5' -AACTTCCTACTACGACCGACG-3'. PCR conditions: 94 x 3min., 92 x 20sec., 63 x 30sec., 72 x 30 sec. (5 cycles), 92 x 20sec., 60 x 30sec., 72 x 30 sec., (35 cycles), 72 x 5min. To amplify the promoter area for sequencing: PCR conditions: 94 x 3min., 92 x 20sec., 55 x 30sec., 72 x 60 sec. (5 cycles), 92 x 20sec., 58 x 30sec., 72 x 60 sec., (30 cycles), 72 x 5min. PCR fragments were subcloned into pZERO1.0 and at least four individual clones/fragment were sequenced to determine methylation frequency.

Statistical significance was calculated using 2 sided Fisher Exact tests.

References

1. Krop, I. E, Sgroi, D, Porter, D. A, Lunetta, K. L, LeVangie, R, Seth, P, Kaelin, C. M, Rhei, E, Bosenberg, M, Schnitt, S, Marks, J. R, Pagon, Z, Belina, D, Razumovic, J, and Polyak, K. HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells. Proc. Natl. Acad. Sci. U S A, *98:* 9796-9801, 2001.

2. Porter, D, Lahti-Domenici, J, Torres-Arzayus, M, Chin, L, and Polyak, K. Expression of high in normal-1 (HIN-1) and uteroglobin related protein-1 (UGRP-1) in adult and developing tissues. Mech. Dev., *114:* 201-204, 2002.

3. Niimi, T, Keck-Waggoner, C. L, Popescu, N. C, Zhou, Y, Levitt, R. C, and Kimura, S. UGRP1, a Uteroglobin/Clara Cell Secretory Protein-Related Protein, Is a Novel Lung-Enriched Downstream Target Gene for the T/EBP/NKX2.1 Homeodomain Transcription Factor. Mol. Endocrin., *15:* 2021-2036, 2001.

4. Minoo, P, Su, G, Drum, H, Bringas, P, and Kimura, S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. Dev. Biol., *209:* 60-71, 1999.

5. Niimi, T, Copeland, N. G, Gilbert, D. J, Jenkins, N. A, Srisodsai, A, Zimonjic, D. B, Keck-Waggoner, C. L, Popescu, N. C, and Kimura, S. Cloning, expression, and chromosomal localization of the mouse gene (Scgb3a1, alias Ugrp2) that encodes a member of the novel uteroglobin-related protein gene family. Cytogenet. Genome Res., *97:* 120-127, 2002.

6. Warburton, D, Schwarz, M, Tefft, D, Flores-Delgado, G, Anderson, K. D, and Cardoso, W. V. The molecular basis of lung morphogenesis. Mech. Dev., *92:* 55-81, 2000.

7. Fujii, T, Dracheva, T, Player, A, Chacko, S, Clifford, R, Strausberg, R. L, Buetow, K, Azumi, N, Travis, W. D, and Jen, J. A preliminary transcriptome map of non-small cell lung cancer. Cancer Res., *62:* 3340-3346, 2002.

8. Garber, M. E, Troyanskaya, O. G, Schluens, K, Petersen, S, Thaesler, Z, Pacyna-Gengelbach, M, van de Rijn, M, Rosen, G. D, Perou, C. M, Whyte, R. I, Altman, R. B, Brown, P. O, Botstein, D, and Petersen, I. Diversity of gene expression in adenocarcinoma of the lung. Proc. Natl. Acad. Sci. U S A, *98:* 13784-13789, 2001.

9. Bhattacharjee, A, Richards, W. G, Staunton, J, Li, C, Monti, S, Vasa, P, Ladd, C, Beheshti, J, Bueno, R, Gillette, M, Loda, M, Weber, G, Mark, E. J, Lander, E. S, Wong, W, Johnson, B. E, Golub, T. R, Sugarbaker, D. J, and Meyerson, M. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc. Natl. Acad. Sci. U S A, *98:* 13790-13795, 2001.

10. Zochbauer-Muller, S, Gazdar, A. F, and Minna, J. D. Molecular pathogenesis of lung cancer. Annu. Rev. Physiol., *64:* 681-708, 2002.

11. Travis, W. D. Pathology of lung cancer. Clin Chest Med, *23:* 65-81, viii,2002.

12. Ross, J. A. and Rosen, G. D. The molecular biology of lung cancer. Curr. Opin. Pulm. Med., *8:* 265-269, 2002.

13. Herman, J. G, Graff, J. R, Myohanen, S, Nelkin, B. D, and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. U S A, *93:* 9821-9826, 1996.

14. Wong, T. S, Kwong, D. L, Sham, J. S, Tsao, S. W, Wei, W. I, Kwong, Y. L, and Yuen, A. P. Promoter hypermethylation of high-in-normal 1 gene in primary nasopharyngeal carcinoma. Clin. Cancer. Res., *9:* 3042-3046, 2003.

15. Toyooka, S, Toyooka, K. O, Maruyama, R, Virmani, A. K, Girard, L, Miyajima, K, Harada, K, Ariyoshi, Y, Takahashi, T, Sugio, K, Brambilla, E, Gilcrease, M, Minna, J. D, and Gazdar, A. F. DNA methylation profiles of lung tumors. Mol. Cancer Ther., *1:* 61-67, 2001.

16. Adorjan, P, Distler, J, Lipscher, E, Model, F, Muller, J, Pelet, C, Braun, A, Florl, A. R, Gutig, D, Grabs, G, Howe, A, Kursar, M, Lesche, R, Leu, E, Lewin, A, Maier, S, Muller,

V, Otto, T, Scholz, C, Schulz, W. A, Seifert, H. H, Schwope, I, Ziebarth, H, Berlin, K, Piepenbrock, C, and Olek, A. Tumour class prediction and discovery by microarray-based DNA methylation analysis. Nucleic Acid Res., *30:* e21, 2002.

17. St Croix, B, Rago, C, Velculescu, V, Traverso, G, Romans, K. E, Montgomery, E, Lal, A, Riggins, G. J, Lengauer, C, Vogelstein, B, and Kinzler, K. W. Genes expressed in human tumor endothelium. Science, *289:* 1197-1202, 2000.

Figure legends

Figure 1. HIN-1 expression and protein levels in human lung and body fluids. A. mRNA in situ hybridization with anti-sense probe (red staining) shows high and specific HIN-1 expression in a subset of bronchial epithelial cells and sparsely scattered reactive type II cells in the lung parenchyma. No signal is detected with the sense probe. **B.** HIN-1 immuno blot analysis of pre-immune (P) or HIN-1 (H) immunoprecipitates of saliva, bronchial wash, plasma, and serum from several independent individuals. The endogenous HIN-1 protein migrates as a smear at ~10 kDa and is highly abundant in all these body fluids. **C.** mRNA in situ hybridization of lung adenocarcinomas and squamous tumors shows high HIN-1 expression in normal bronchial epithelial cells in tumors 256 and 57 (arrows), and in epithelial cells of well-differentiated parts of the two adenocarcinomas. Adjacent moderately differentiated areas of the two adenocarcinomas and all squamous tumor cells lack HIN-1 expression. **D.** RT-PCR analysis of HIN-1 expression in different parts of normal prostate (LPZ-left peripheral zone, RPZ-right peripheral zone, and CZ-central zone), benign prostatic hyperplasia (BPH), and prostate carcinomas (T1-7). High HIN-1 expression is detected in all areas of the normal prostate and in BPH, while most prostate tumors lack HIN-1 expression. Amplification of the β-actin gene was used as control.

Figure 2. Analysis of methylation patterns of the HIN-1 proximal promoter region and first exon in various cell and tissue types. A. Results of sequence analysis of bisulfite treated genomic DNA from the indicated cell and tissue types. ZR75-1-AC indicates 5azaC treated cells, whereas BEC refers to primary bronchial epithelial cells. Circles represent potential methylation sites (CpG), while black and white coloring indicates the frequency at which the site was found to be methylated in the clones analyzed (○-0 %, ◔-25%, ◑-50%, ◕-75%, ●-100%). HIN-1 mRNA levels are indicated by "+" and "-" signs; "+++" denotes high level of expression detected in normal mammary epithelial cells, "+"

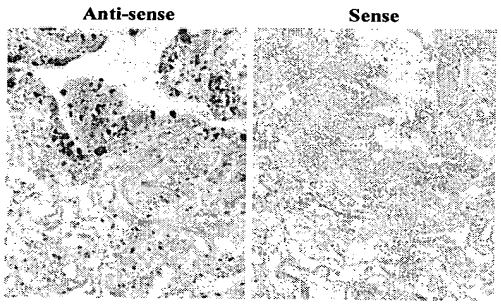
indicates mRNA levels detectable by Northern blot analysis of 5 μg of total RNA, while "+/-" in the case of SUM225 cells indicates variable results obtained in early (some HIN-1 expression) and later (no HIN-1 expression) passage cells. The methylation result depicted was obtained using genomics DNA from later passage cells. **B.** Methylation specific PCR analysis of the HIN-1 promoter region in lung tumors (T) and corresponding non-malignant (N) lung tissue, and normal lung obtained from hamartoma patients (N1-N4). M and U indicate amplification using methylated and un-methylated sequence specific primers, respectively.

Table 1. HIN-1 promoter methylation in various normal and cancerous tissues

Tissue	Sample size	Unmethylated No. (%)	Methylated No. (%)
Lung			
NSCLC (squamous and adenocarcinomas) Normal	28	18 (64)	10 (36)
NSCLC (squamous and adenocarcinomas) Tumor	28	14 (50)	14 (50)
NSCLC Squamous Tumor	12	3 (25)	9 (75)
SCLC Normal	16	15 (94)	1 (6)
SCLC Tumor	16	13 (80)	3 (20)
Cancer cell lines	6	2 (33)	4 (67)
Prostate			
Normal	38	11 (29)	27 (71)
Cancer	21	1 (5)	20 (95)
Xenografts	9	0 (0)	9 (100)
Pancreas			
Normal	12	5 (42)	7 (58)
Cancer	17	8 (47)	9 (53)
Cancer Cell Lines	10	0 (0)	10 (100)

HIN-1 promoter methylation was evaluated by MSP. Both partially and completed methylated samples are listed together as methylated. NSCLC stands for Non-small cell lung cancer, whereas SCLC indicates small cell lung cancer.

A



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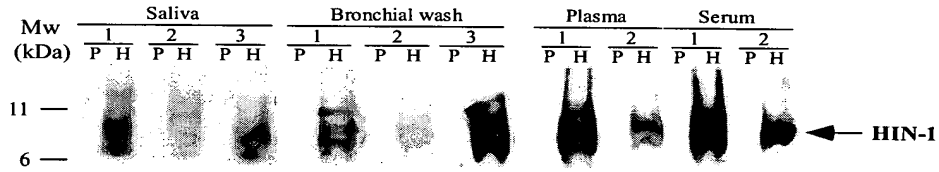
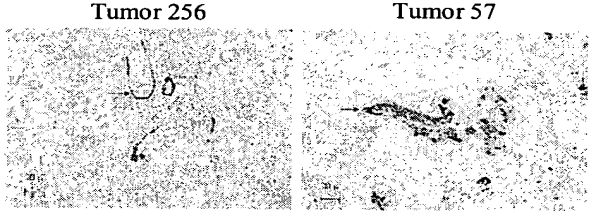
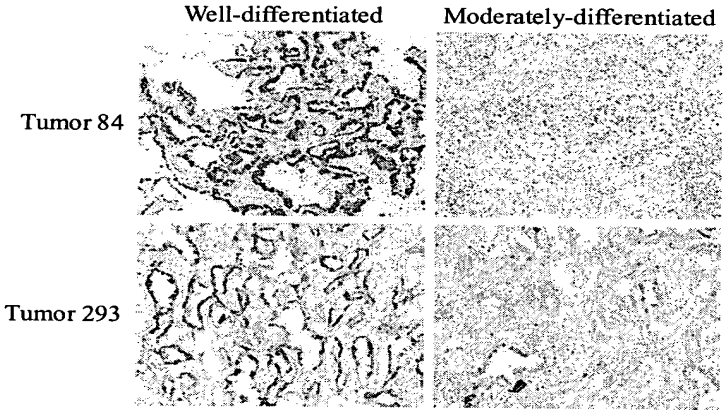


C

Squamous tumors



Adenocarcinomas



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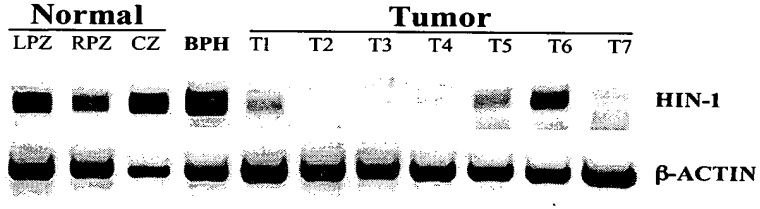

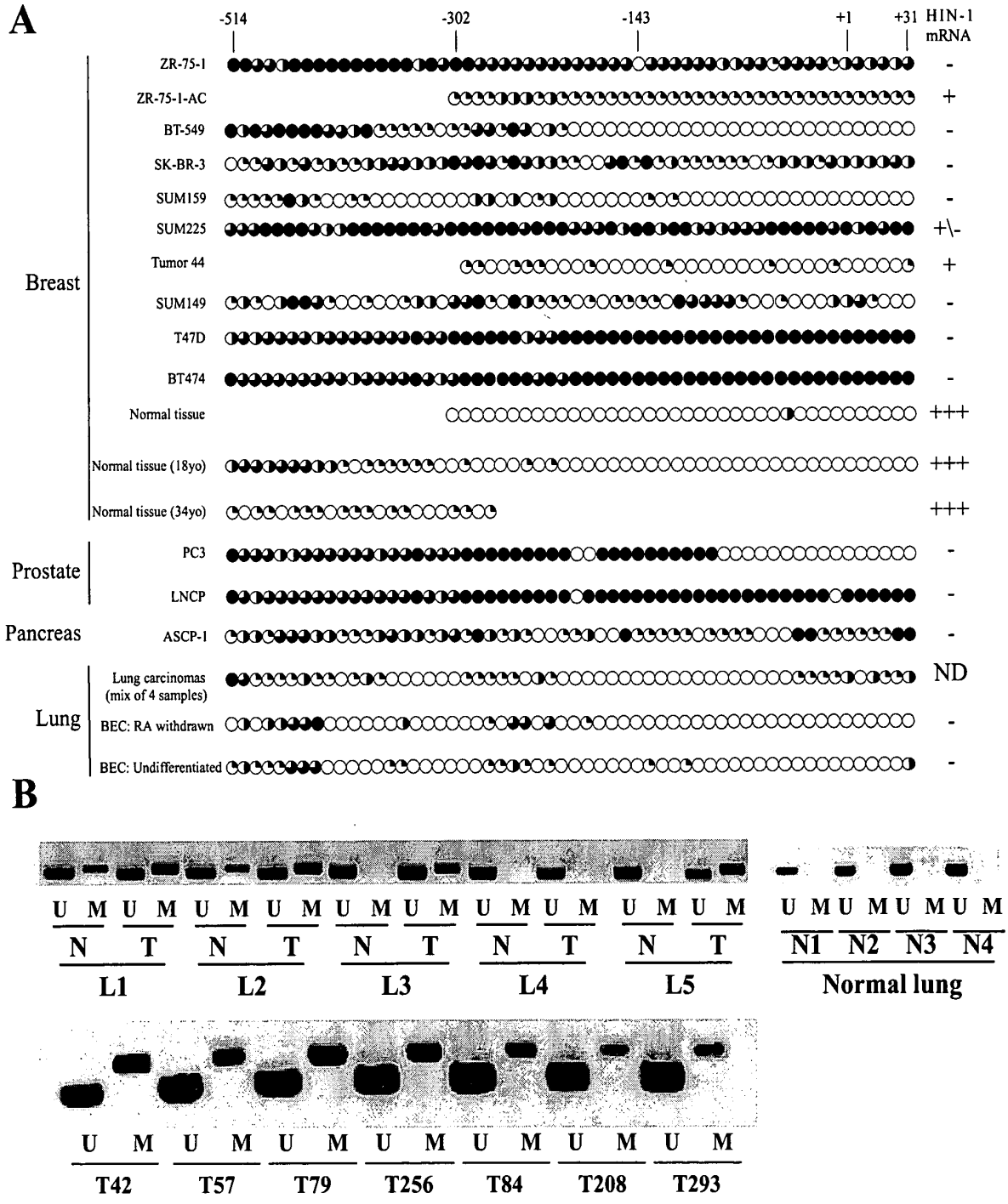
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SUM225
Tumor 44
SUM149
T47D
BT474
Normal tissue
Normal tissue (18yo)
Normal tissue (34yo)
Prostate
PC3
LNCP
Pancreas
ASCP-1
Lung
Lung carcinomas (mix of 4 samples)
BEC: RA withdrawn
BEC: Undifferentiated
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